

European Language Institute

Founded 1938

3844 Carnation Circle South
Palm Beach Gardens, FL 33410-5636
Tel.: (561) 622-1442 Fax.: (561) 622-3161

World Organisation for Intellectual Property
International Office

PCT

Seal

**International Registration as per agreement on
international cooperation in the field of patents (PCT)**

(51) International patent classification 6: G01N 33/68

A 1

(11) International publication number: WO 98/07036

(43) International date of publication: February 19, 1998

(21) International file number: PCT/EP97/04395

(22) International filing date: August 13, 1997

(30) Priority dates: 196 32 521.8 August 13, 1996 DB (DE?)
197 25 362.8 June 16, 1997 DB (DE?)

(71)(72) Filing person & Inventor: Wolf-Georg Forssman, Hannover

(72) Inventor, and (75) Inventor/Filing person (only for US):

Peter Schulz-Knappe, Hannover; Michael Schrader, Hannover; Hans-Georg Opitz, Hannover

(74) Attorneys: Hans-Wilhelm Meyers etc, Köln

(81) Designated states: AL,AU,...(mostly illegible, pls compare with original)

Published: With International Research Report

Before expiration of allowed term for changes in claims; publication will be repeated when changes arrive.

(54) Title: PROCESS FOR DETERMINING THE STATUS OF AN ORGANISM BY PEPTIDE
MEASUREMENT

(57) ABSTRACT: A process is disclosed for determining the status of an organism by measuring peptides in a sample of the organism which contains high-molecular and low-molecular peptides and acts as an indicator of the organism status. Low-molecular peptides are directly sensed and characterized, and are then correlated with a reference.

Note by translator:

For partly illegible full addresses of persons mentioned, please compare with original.

European Language Institute

Founded 1938

3844 Carnation Circle South
Palm Beach Gardens, FL 33410-5636
Tel.: (561) 622-1442 Fax.: (561) 622-3161

WO 98/07036

PCT/EP97/04396

Procedure to register the status of an organism by measuring the peptides.

The subject of the invention on hand is a procedure to understand the status of an organism by measuring the peptides in a sample of that organism.

For the understanding of the status of an organism various analytical methods are applied. For example, in the diagnostics of higher organisms during pathological diagnoses it has been tried to fathom the cause of the pathological change on the basis of symptomatology in order to develop a causal therapy. It has further been endeavored to develop a reference of an average 'healthy' organism by sequencing the genomes of organisms and the establishment of 'wild type genomes', in order then to discover individual deviations by appropriate gene analyses, which can indicate possible pathogenic developments. The first methodical inception is prejudicial in as much as no diagnosis can be done without hypotheses, since in this a diagnosis is undertaken which is already based upon suppositions. The second procedure is detrimental, because at long sight it will not as yet be possible to diagnose important or even all illnesses which can be traced back to genetic malfunctions. A further disadvantage of the last mentioned method may also lie in the fact, that a mutation in a gene does not unconditionally lead to the expression of the thereby connected phenotype.

It would thus be desirable, to have at disposal a universally applicable diagnostic procedure, by which the mentioned disadvantages can be successfully avoided and in particular, a diagnosis free of hypotheses can be executed. The diagnostic procedure should further be universally applicable, and not be limited to higher developed systems, but be equally useful to grasp the status of low organisms. It should further be easily installed and be possible to execute the procedure with basically known techniques.

A technical problem which lies on the bottom of this invention is thus the provision of such a procedure.

Surprisingly, the underlying technical problem of the invention is solved in a simple way by the procedure with the features of claim 1. The subordinated claims concern preferred forms in execution of the procedure in accordance with this invention.

In conformity with this invention, the procedure for comprehending the status of an organism emanates from taking a specimen of the organism to be examined. The experiment can also be the complete organism. The test must contain low molecular peptides, whereas it does not interfere, if the specimen also contains high molecular peptides or proteins besides the low molecular peptides. The low molecular peptides, according to the invention, are grasped at this directly, characterized and serving as indicator for the status of the organism. At this it is possible as well, to grasp by the technique of measuring individual peptides directly, to collect by the technique of measuring more peptides and do so until all low molecular peptides are collected which are contained in the specimen and which can be grasped by the technique of measuring. Different than by the traditional analytic or diagnostic methods, like for example the gel-electrophoresis or the two-way electrophoresis and for example the clinical diagnostic methods, here the high molecular structures are not examined, like for instance the proteins. Contrary to basically known diagnostic methods like for example radioimmuno-assay or other competition-assays for the measuring of peptide hormones and similar, the low molecular peptides are, as per this invention, collected directly by the measuring technique, and not indirectly as in the mentioned methods. The distribution of low molecular peptides serves as reference in a representative cross section of defined controls.

In the procedure of the invention, the specimen of tissue or liquid tests to be examined and of which the status is to be measured, can be derived from the organism or it could be the organism itself or parts of it. In the case of examination of lower organisms, the organism itself serves preferably as specimen. In particular, protozoons are considered low organisms, like procarcyote systems or simple eucaryote systems, like yeast or other micro-organisms.

In accordance with the invention, the low molecular peptides, which are pulled in for the measuring, should preferably show a molecular weight of maximal 30,000 Dalton. The lower margin in itself is not critical, however, dipeptides represent the

low frontier of the low molecular peptides which must be collected according to the invention. Particularly preferred are the molecular weights of the low molecular peptides from 100 to 10,000 Dalton.

If required, for example when needed because of a changed measuring layout, it can be of advantage to remove high molecular peptides or proteins from the specimen as well as other biopolymers, which potentially can interfere with the measurement. In particular, this is not required, when by the application of the measuring method according to the invention, the higher molecular peptides are not included in the measuring technique.

In the invention, the mass spectroscopy is preferably applied to seize the low molecular peptides. The so-called MALDI-method (matrix supported laser-desorption-ionisation-mass-spectroscopy) has proven particularly successful in this. When the mass-spectrometry is installed as method, it is recommended to use the data which can be determined by the mass-spectroscopy for the characterizing of the low molecular peptides, like, for example, their molecular weight. It is likewise possible, to analyze other parameters under certain circumstances, like, for example, the loading of the peptides or the characteristic time of retention of chromatographic columns, or a fragment pattern of the low molecular peptides, or combinations of mass of the low molecular peptides and their loadings.

Depending upon the statement of the problem, which is also connected with the gathering of the status of the organism, it may be of advantage, to distribute the specimen into several fractions and to analyze the samples by different formulations of the question or by a different layout of the measuring technique and thus to gather the status of the organism.

As organism serve in particular procaryote, eukaryote, multicellular organisms, cells of tissue cultures, cells of animals and humans. Thus it is possible through this invention, to examine the status of genetically altered or transformed and/or conditioned organisms. This can be of special advantage when examining transformed systems, in order to recognize to what extent transformed organisms have developed possibly unexpected or unwanted features, whilst, for example, peptides are formed, which indicate unwanted or unexpected properties, like toxic features. Noticibly, any deliberate or unawarely undertaken manipulation (conditioning) of an organism can influence its status, be it within the framework of administering medications, the genetherapy, with infections, at the place of work by

contact with chemical materials, with laboratory animals, in particular with transgene animals and knock-out mutants. Especially with such procedures, it can be scrutinized by intra- and inter-individual comparisons, for example by chronologic collection of specimens from the organism before and during the duration of one of the above mentioned measures, or by comparison with untreated control organisms, whether predicted, desirable changes in status have in fact occurred, and whether, moreover or instead of it not forecast, undesirable or even desirable alterations have entered, which are collected by the procedure of the invention, free of hypotheses.

For this reason the procedure of the invention is also suited for example to accompany clinical studies, toxicologic examinations, when testing medicines of all kinds, for the analysis / gathering of decomposition products, and for the analysis of gene products.

In the veterinary- and human medical science the procedure of the invention evolves into its surpassing importance, by making it feasible to gather, without hypotheses the status of the organism in question. Therefore no confirmation assay is executed with an already preconceived opinion, but moreover a genuine total picture of the status of the examined organism becomes available. The procedure of the invention, which can be described as differential peptide display, emanates in this from that in a healthy organism there is a certain peptide pattern, which is therefore in the position to serve as reference standard. Taking now the peptide status of an individual and comparing it with the reference, one can on the one hand determine deviations, which already give a first indication of a possible photogenous condition. When these deviations, which have been established by comparison with similar photogenous conditions, are established from appropriate specimens of a sick person, the disease in question can already be directly identified from the analysis by comparison of the deviations in the peptide pattern of the specimen of the individual and by conformity of the deviation with an assigned clinical picture.

In accordance with the invention one can in particular proceed with this as follows: For the creation of a reference specimen one can use above all ultra filtrates of body fluids and tissue extracts. The collection of filtrate peptides and their fractionation into fractions is done whilst, for example, low molecular peptide fractions are extracted. The characterizing of the peptide fractions can be done, for example, by means of retention behavior and molecular mass, ascertainable by chromatography or mass spectroscopy. If, for example, ultra filtrates of patients are

utilized, who suffer from a known disease, and these are compared with the previously established spectrum of healthy reference test subjects, an assignment of the specific disease with the status of the peptide mixture in question can be accomplished according to the deviation of the pattern. The method by itself thus can also be applied in traditional ways, in that for example the appropriate peptide sample which indicates pathogenic changes, is directly scrutinized. In special cases this could even be a characteristic peptide for the appropriate disease. When a specimen of a patient is analyzed, for example, with whom a certain syndrome is discernible, and there exists a hypothesis for the cause of this disease, this specific peptide, for example, can also be scrutinized in the analysis, according to the invention, and with positive results the appropriate therapy plans can be established. It is entirely possible, to extract first a specimen from the patient, and to establish a status with the procedure of the invention, in order then, while determining the presence of a deviation indicating pathogenic conditions, to perform a control measuring either by basically known confirmation assays, by employing the usual clinical assays, or to do it by control measuring through specific screening as per the indicator of the pathogenic condition.

Peptides could be extracted in this, after a procedure which is known to the expert, like for example, by ultra filtration of the appropriate source. For this filters are used with a molecular exclusion size which lie within the range required in accordance with the invention, thus between one Dipeptide and maximal 30,000 Dalton. By suitable choice of the respective membranes, certain molecular weight fractions can also be extracted. Preferably 0.2 ml to 50 l filtrate are extracted in the frame of the filtration, which, for example, immediately after the termination of the filtration is stabilized to a pH value of 2-4 by acidulation with diluted hydrochloric acid. The mentioned quantities serve in particular to examine pooled specimens, on one side to develop reference samples of healthy probands, respectively to determine specific disease peptide markers to establish a peptide data bank.

The peptides, which are available in the filtrate after ultra filtration are extracted by absorption onto chromatographic materials, above all the cation exchanger, as for example fracto gel, anion-exchange resin fracto gel TMAE and reverse phase (RP) materials, with subsequently following elution by linear gradients or by shoulder gradients. For the further purification, if need be, further chromatographic isolations, in particular over RP-phase material, can be done.

The collecting of the peptide fractions is preferably done by mass spectrometric analysis, in particular with the MALDI-MS (matrix assisted laser desorption ionisation mass spectrometry), or the ESI-MS (electro spray ionisation-MS). These are methods which can be applied for the analysis of peptides. With this it is preferably worked with an on-line coupling of a micro bore RP-isolation and the mass spectrometry (LC-MS-coupling). From the gained data a multidimensional table by retention behavior, molecular weight, and signal intensity, as preferred guide parameter, is created. Also other readings, however, which become available with the mentioned methods can be gathered.

The data gained from patients with a known basic disease, via the above mentioned steps, are compared with the data which were gathered in the same way from a healthy reference population. With this, both qualitative changes (for example the appearance of new peptides or the missing of peptides), as well as quantitative changes (the increased, respectively the decreased appearance of individual peptides) are ascertained. The targets, which were defined by the comparing analysis, can, if needed, be furthermore purified and identified peptide chemically by methods which are known to the expert. The received sequence information can then be compared with protein- and nucleic acid data banks and subsequently also with literature data. The relevance of the described peptides, in reference to the scrutinized disease, is examined by functional studies and by serial screening of suitable patient groups.

Example 1

Use of body fluids, here: Blood filtrate (Hemo filtrate, HF)

HF is executed by techniques which are known to the expert in the frame of an arterial-venous or also venous hemo filtration on selected patients or probands. The extraction of HF is done in the way, as it is principally and routinely done on patients, with chronic nephropathy. Through an arterial drainage and venous afference (arterial-venous HF) or a venous drainage with venous afference (veno-venous HF), the blood of the patient is - with apparatus support - channeled through a hemo filter device (for example Hemo Processor, Sartorius, Göttingen; AK 10 HFM, Gambro, Hechingen) over a hemo filter (for example Hemoflow F 60 or Hemoflow HF 80 S, Fresenius, Bad Homburg; Hemoflow FH 77 H and Hemoflow HF 88 H, Gambro), which has a molecular exclusion size of up to 30 kDa. The filtrate volume which was withdrawn from the patient is replaced by an electrolyte solution (for example SH 01,

SH 05, SH 22, SH 29, Schiwa, Glandorf).

In the frame of the procedure on hand a diagnostic hemo filtration is executed with the goal, to collect between 1 and 30 l HF from a patient within a hemo filtration. The hemo filtrate, to avoid proteolysis, is immediately adjusted to a pH value between 2 and 4 with diluted acid (for example 1 M HC1), and cooled down to 4°C.

2. Collecting of HF-peptides and undoing into fractions.

2.1 Peptide extraction with gradual elution.

10 l hemo filtrate is thinned down to a conductivity of 6 mS/cm with deionised water and the pH adjusted to 2.7 with hydrochloric acid. The HF is then applied on to a chromatographic column. After bonding of the HF-peptides, the bonded peptides are eluted with a pH graduated elution. In this, 7 buffers with ascending pH are employed.

Chromatography requirements:

Flow at filling:	100 ml/min		
Flow at eluting:	30 ml/min		
Detection:	214, 280 nm		
Column:	Vantage (Amicon, Witten), diameter 6 cm x 7 cm filling height		
Column material:	Fraktogel TSK SP 650 M (Merck, Darmstadt)		
Apparatus:	BioCAD 250, Perseptive Biosystems, Wiesbaden-Nordenstadt		
Buffer	pH	Buffer Substances	Molarity
Elution buffer 1	3.6	citric acid	0.1
Elution buffer 2	4.5	acetic acid	0.1
Elution buffer 3	5.0	malic acid	0.1
Elution buffer 4	5.6	succinic acid	0.1
Elution buffer 5	6.6	sodium dihydrogen phosphate	0.1
Elution buffer 6	7.4	disodium hydrogen phosphate	0.1
Elution buffer 7	9.0	ammonium carbonate	0.1

The eluates 1-7 are collected separately

2.2 Second chromatographic undoing

The eluates 1 - 7 are chromatographed separately over a reverse phase column.

Chromatographic requirements:

Flow at filling: 10 ml/min

Flow at eluting: 4 ml/min

Detection: 214 nm

Column: HPLC-Steel column, diameter 1cm, filling height 12.5

Column material: Source RPC 15 μm (Pharmacia, Freiburg)

Apparatus: BioCAD, Perseptive Biosystems, Wiesbaden-Nordenstadt

The eluate is collected in 4 ml-fractions.

3. The mapping (filing?) of the peptide-fractions

3.1

Aliquots of the collected fractions in 2.2 are spread on a microbore-reverse-phase-column and eluted in gradient. The detection is done with a UV-detector and on-line with an electrospray-mass-spectrometer.

Chromatographic requirements:

Flow at filling: 20 $\mu\text{l}/\text{min}$

Flow at eluting: 20 $\mu\text{l}/\text{min}$

Detection: 220 nm

Column: C18 AQS, 3 μm , 120 Å, diameter 1 mm, length 10 cm (YMC, Schermbeck)

Apparatus: ABI 140 B Dual solvent delivery system

Buffer A: 0.06% trifluor acetic acid in water

Buffer B: 80% acetonitrile in A

Gradient: 0% B on 100% B in 90 min

On-line mass spectrometry:

API III with electrospray -interface (Perkin-Elmer, Weiterstadt)

Positive Ion Modus

Measuring range: m/z from 300 to 2,390

Scan-time: 7 sec.

Scan-window: 0.25 m/z

Data collecting is done with MacSpec or MultiView software (Perkin-Elmer).

3.2 MALDI-MS measuring of the individual fractions

Aliquots of the fractions which were collected in 2.2 are measured with variable matrix substances, for example with additive of L (-) fucose in MALDI-MS.

From the crude data a multidimensional chart is established with regard to the scan number, signal intensity, and after calculation of the masses from the multiply loaded ions of a scan.

4. Comparative analysis

4.1 Identification of new, missing, or in their mass clearly different peptides

By comparing the under 3.3 preserved data statements, which can also be called peptide chart, qualitative and/or quantitative differences can be determined. In this, in regard to controls and specimens, individual data records or also groups of data records are drawn upon for comparison.

4.2 Peptide chemically characterizing the identified targets.

Of the collected raw material (for example bulk preparations of hemo filtrates) the identified targets are cleaned up in quantities, which permit identification. For this, as known to the expert, the various chromatographic separation techniques (reverse phase, ion exchange, size exclusion chromatography, hydrophobic interaction chromatography, etc.) are applied, as they are generally implemented to undo peptide mixtures. After every chromatographic separation of a fraction, the targets are once more identified over ESI-MS, MALDI-MS or also LC-MS in the fractions. This procedure is repeated under variation of the chromatographic parameters so many times, until a clean product of the sought after specification, i.e., retention time and molecular mass, is on hand. After this the definition of a partial- or complete amino acid sequence or fraction pattern follows. In addition a data bank comparison is done with the known data banks (Swiss-Prot and EMBL-Peptide- and Nucleic acid - data bank) with the aim of identification of the partial- or complete sequence or of a fraction pattern. If no data bank entry exists, the clarification of the primary structure follows.

Example 2:

Use of body fluids, here: ascites

1. Collection of ascites

Ascites are formed as extra vasal exudate during various diseases (malignant tumors, liver malfunctions etc). In the frame of the procedure on hand, between 10 ml and 10 l of ascites are collected by puncture, and then to avoid proteolysis immediately adjusted with diluted acid (for example 1 M HCl) to a pH value between 2.0 and 4.0 and cooled down to 4°C. After ultra filtration over a cellulose triacetate membrane with an exclusion size of 30 kDa (Sartocon-Mini-Apparatus, Sartorius), the filtrate is used as source of peptides from there on.

2. Extraction of ascite-peptides and separation in fractions

2.1. Peptide extraction with gradient-elution

5 l ascite filtrate is adjusted to pH 2.0 and separated over a preparative reverse - phase- column.

Chromatographic requirements:

Flow at filling: 40 ml/min
Flow at eluting: 40 ml/min
Detection: 214 nm, 280 nm
Column: Waters cartridge system, diameter 4.7cm, filling height 30cm
Column material: Vydac RP-C18, 15 - 20 μ m
Apparatus: BioCAD, Perseptive Biosystems, Wiesbaden-Nordenstadt
Buffer A: 0.1% trifluorine acetic acid in water
Buffer B: 80% acetonitrile in A
Gradient: 0% B to 100% B in 3,000 ml

The eluate is collected in 50 ml fractions.

The further course of characterizing corresponds with example 1.

Example 3:

Use of body liquids, here: urine

1. Collection of urine

Urine is collected directly as catheter specimen or as spontaneous urine of 0.5 to 50 l, and to avoid proteolysis immediately adjusted to a pH value of between 2.0 and 4.0 with diluted acid (for example 1 M HC1) and cooled down to 4°C. After ultra filtration over a cellulose triacetate membrane with an exclusion size of 30 kDa (Sartocon-Mini-apparatus, Sartorius) the filtrate is used as source of peptides from there on.

2. Collection of urine-peptide and separation into fractions

2.1 Peptide extraction with graduated elution

10 l urine-filtrate are diluted with water to a conductivity of 6 mS/cm and the pH value adjusted to 2.7 . The urine-filtrate is then spread upon a chromatographic column. After binding of the peptides, the bound peptides are eluted with a common salt gradient.

Chromatographic requirements:

Flow at filling: 100 ml/min
Flow at eluting: 30 ml/min
Detection: 214 nm
Column: Vantage (Amicon, Witten) diameter 6cm x 7 cm filling height
Column material: Merck Fraktogel TSK SP 650 M
Apparatus: BioCAD 250, Perseptive Biosystems, Wiesbaden-Nordenstadt
Buffer A: 50 mM NaH₂PO₄ pH 3.0
Buffer B: 1.5 M NaCl in A
Gradient: 0% B to 100% B in 2,000 ml

The eluate is collected in 10 pools á 200 ml.

2.2 second chromatographic separation

The fractions are chromatographed separately over a reverse-phase- column.

Chromatographic requirements:

Flow at filling: 10 ml/min
Flow at eluting: 4 ml/min
Detection: 214 nm
Column: HPLC steel column, diameter 1cm x 12.5 cm filling height
Column material: Pharmacia source RPC 15 µm
Apparatus: BioCAD, Perseptive Biosystems, Wiesbaden-Nordenstadt

Buffer A: 0.1% trifluorine acetic acid in water
Buffer B: 80% acetonitrile in A
Gradient: 0% B to 100% B in 200 ml

The eluate is collected in 4 ml fractions.

The further course of characterizing corresponds with example 1.

C l a i m s

1. Procedures to record the status of an organism by measuring the peptides of a specimen of the organism, which contains high- and low molecular peptides, as indicator for the status of the organism, whereas
 - low molecular peptides are directly collected and characterized,
 - and are placed in relation to a reference.
2. Procedures as per claim 1, whereas the specimen, tissue- or fluid samples, are from the organism, or are the organism itself, or combinations of both.
3. Procedures as per claim 1 and/or 2, whereas the low molecular peptides, which are drawn upon for measuring, show a molecular weight of maximal 30,000 Dalton.
4. Procedures as per claim 3, whereas the low molecular peptides, which are drawn upon for measuring, show at least a molecular weight which corresponds to that of the dipeptides.
5. Procedures as per claim 3 and/or 4, whereas the low molecular peptides, which are drawn upon for measuring, show a molecular weight of between 100 and 10,000 Dalton.
6. Procedures as per at least one of the claims 1 to 5, whereas the high molecular peptides are separated before the measuring of the low molecular peptides, or are in the technique of measuring or in the technique of evaluation not taken into consideration.

-14-

7. Procedures as per at least one of the claims from 1 to 6, whereas the collection of the low molecular peptides is done by mass spectrometry.
8. Procedures as per at least one of the claims from 1 to 7, whereas the low molecular peptides are characterized by measuring their molecular weight.
9. Procedures as per at least one of the claims from 1 to 8, whereas the specimen before measuring the low molecular peptides is divided into various fractions and measured under different conditions.
10. Procedures as per at least one of the claims from 1 to 9, whereas prokaryotes, eukaryotes, multicellular organisms, cells from tissue cultures, cells from animals and humans serve as organisms.
11. Procedures as per at least one of the claims from 1 to 10, whereas the specimen originates from genetically changed or transformed, and/or conditioned organisms.
12. Procedures as per at least one of the claims from 1 to 11, whereas the understanding of the status of the organism serves for the examination, without hypotheses, and mapping (picture) of the status of the total organism, to discover possible deviations from a reference condition.
13. Procedures as per at least one of the claims from 1 to 11, whereas the understanding of the status of a transformed organism serves for the examination, free of hypotheses, and mapping (picture) of the status of the total organism to discover mutations of the transformed organism, to discover the appearance of peptides in connection with the transformation which are causally connected with changes in metabolic processes.